REMARKS

Claims 49 through 77 are pending in this application. No amendment is made to the claims in this Response.

Claims 49-77 are rejected under 35 U.S.C. 112, first paragraph, because the specification is not enabling for methods of differentiating any of "two types" of thyroglobulins and methods of diagnosing cancer (Examiner's point 3).

The rejection of claims 49-77 as not being enabled is respectfully traversed. Applicants believe that the specification provides both "written description" and "enablement" of the claims, as required by 35 U.S.C. 112, first paragraph.

The Examiner states that the specification does "does not reasonably provide enablement for methods of differentiation any of "two types" of thyroglobulins[']] and methods of diagnosing cancer. The specification does not enable any person skilled in the art ... to practice the invention commensurate in scope with these claims." The Examiner further discusses the rejection on p. 3 of the Office Action, stating ".. however, sorting thyroglobulins by sugar chain variations would not differentiate any "type" of thyroglobulin, by [sic] rather only those that have a detectable difference in their sugar chain structure."

In traversing the rejection, Applicants first note that there is no indefiniteness or written description issue with regard to the claims. Claim 49 and the other claims clearly state what is meant by "two types of thyroglobulin." Claim 49, for example, distinguishes between "a first type of thyroglobulin" and "a second type of thyroglobulin," and defines the meaning of these types in the

claim. Applicants believe that this definition of the types of thyroglobulin is fully supported by the specification.

The first enablement issue raised by the Examiner appears to center on "methods of differentiating any of "two types" of thyroglobulin." However, the Examiner's remarks on page 3, lines 9-15 are not clearly directed to an enablement issue. Line 12 states: "Any 'type' of thyroglobulin could refer to other non-sugar chain modifications, sequence variations, or even production in distinct species, etc. Detection of sugar chain molecule variations would not be indicative of the broadly claimed 'types' of thyroglobulins." Applicants are uncertain that these remarks are directed to enablement.

As best understood by the Applicant, the Examiner is stating that differences in the sugar structures of two thyroglobulins does not actually mean these are different "types" of thyroglobulin. Applicants strongly disagree with this position. A thyroglobulin includes a sugar chain, and therefore a difference in the sugar chain yields a different thyroglobulin molecule. In the present claims, Applicants are functionally defining "types" of thyroglobulin based on binding properties of the sugar chain of the thyroglobulin. Applicants may be their own lexicographer in this regard. The definition of "types" of thyroglobulins in the claims would not appear to be an enablement issue.

Enablement of the claim requires that one of ordinary skill in the art be able to practice the claim based on the specification. The Examiner is apparently stating that, in claim 49, for example, one of ordinary skill in the art could not obtain the anti-thyroglobulin antibody used in step (a) or the specific lectin or specific antibody of step (b). (One of ordinary skill in the art could certainly perform the recited steps of adding these compounds.)

Applicants believe that one of ordinary skill in the art could obtain these compounds. The specification makes it clear on page 1 that thyroglobulins are well known in the art, as is the fact that some thyroglobulins have different sugar chains. Anti-thyroglobulin antibodies and lectins are well known in the art. One of ordinary skill in the art can produce a new anti-thyroglobulin antibody to a particular thyroglobulin, if necessary. Therefore, one of ordinary skill in the art could certainly obtain anti-thyroglobulin antibodies and specific antibodies and lectins as recited in the claims, and therefore could carry out the recited methods.

The Examiner also discusses the claims regarding diagnosis of a malignancy, stating: "The claims broadly recite any antibody or lectin, regardless of what it binds, many of which in no way correlate to malignancy and thus would not function unless the appropriate lectins were used." The Examiner here appears to be stating that Applicants' claims are overly broad and encompass examples which would not function for the purpose stated in the preamble.

In response to this, Applicants note the following points. First, Applicants are not certain how correct the Examiner is that some lectins meeting the limitations of the claims would not work for diagnosing cancer, but Applicants do not believe that this is relevant to the issue of enablement. Applicants believe that the claims clearly define a method and that what is at issue is that one skilled in the art could readily perform the recited method. How medically accurate the determination resulting from performing the method is, is not an enablement issue. Applicants believe that one skilled in the art could perform the method, indeed with almost no experimentation necessary, and that the claims are enabled.

Applicants respectfully believe that some of the Examiner's remarks may actually be directed to the issue of utility under 35 U.S.C. 101. However, Applicants have clearly stated a utility and Applicants believe that this utility is not "unbelievable." Applicants note that it is, in fact, almost inevitable that broad claims encompass some modes which do not work as well as others. That is, even if some lectins which meet the limitations of the claims do not produce as good a cancer diagnostic method as others, the recited invention still has utility under 35 U.S.C. 101.

Applicants therefore believe that claims 49-77 are fully enabled.

Claims 49-66, 68-75 and 77 are rejected under 35 U.S.C. 103(a) as unpatentable over Hanham et al. (Biochemica et Biophysica Acta, Vol. 884, 1986) in view of Voller et al. (Rul. World Health Organ., Vol. 53, pp. 55-65, 1976) or Harlow and Lane (Antibodies, a Laboratory Manual, Chap. 14, pp. 553-612, 1988) or Samuel et al. (U.S. Pat. No. 5,242,799) (Examiner's point 4).

The Examiner cites Hanham et al. ("Hanham") as using "an anti-thyroglobulin antibody which is capable of binding to both types of thyroglobulin and further using a lectin ... which is capable of binding a specific sugar chain structure on only one of the two types of thyroglobulin. The method of Hanham et al. measures thyroglobulin using both antibodies and lectins in combination with one another." The Examiner states that the difference between Hanham and the recitation of the claims is the order of the steps.

Applicants respectfully traverse the rejection of claims 49-66, 68-75 and 77, as Applicants believe that no *prima facie* case of obviousness can be made using the cited references. For

simplicity, Applicants will address their remarks to claim 49, but these remarks are applicable to other rejected claims.

First of all, Applicants note that claim 49 differs from Hanham in the following respects:

1) Hanham does not describe adding an antibody or a lectin to a fluid sample containing thyroglobulin, as in step (a) or step (b). Hanham only describes preparation of gels containing an antibody or a lectin through which thyroglobulins are electrophoresed (p. 160, column 2).

2) Step (b) of claim 49 requires that both the anti-thyroglobulin antibody and the lectin have been added to the same fluid sample, so as to be simultaneously in contact with the thyroglobulins. In Hanham, the antibody and lectin are in separate gels of a two-tiered gel, and it does not appear that both the lectin and antibody are ever even simultaneously in contact with the electrophoresed thyroglobulin.

3) Step (c) of claim 49 requires measuring the amounts of conjugates of thyroglobulin. Hanham does not appear to actually measure the amount of any thyroglobulin using the electrophoretic method. Rather, Hanham describes a qualitative analysis of lectin binding using lectin affinity electrophoresis.

The Examiner also cites Samuel et al. ("Samuel") as discussing numerous lectin/antibody assays. The assays include a heterologous sandwich immunoassay using human TF (Thomsen-Friedenrich) erythrocyte antigen as the "catcher" and labeled peanut agglutinin, a lectin, as the "probe" (column 5, line 15). However, Samuel appears to state a distinct purpose of generally replacing antibodies with lectins in an immunoassay (column 3, line 15), and therefore Samuel suggests a lectin only in this regard. More significantly, there appears to be no indication in Samuel

that there may be two types of TF-antigen, one of which does not bind the lectin (to be analogous to the two types of thyroglobulins), and therefore Samuel does not suggest the limitations on the antibodies and lectins recited in the claims.

Moreover, Samuel is clear that the lectin must bind to the antigen independently of the antibody (column 5, line 17). However, claim 60, for example, recites a specific case of use of an antibody which will not bind to the thyroglobulin when the lectin is bound. Samuel clearly teaches away from this method.

The Harlow and Lane reference and Voller et al. are general references which do not address the specific antibodies recited in the claims, and which do not appear to describe lectins. Therefore, these references only disclose general kinds of immunoassays, but do not suggest the specific recitation of claim 49.

Therefore, the primary reference, Hanham, does not suggest adding any antibodies or lectins to a fluid sample. None of the references discloses or suggests the limitations on the antibodies and lectins recited in claim 49 or in the other claims. It would appear to be impossible to construct a *prima facie* case of obviousness using these references, and Applicants believe that claims 49-66, 68-75 and 77 are novel and non-obvious over Hanham et al., Voller et al., Harlow and Lane and Samuel et al., taken separately or in combination.

Claims 49-66, 68-75 and 77 are rejected under 35 U.S.C. 103(a) as unpatentable over Heilig et al. (Endocrin. Suppl. 108(267), p. 151, 1985) in view of Voller et al. (Rul. World Health Organ., Vol. 53, pp. 55-65, 1976) or Harlow and Lane (Antibodies, a Laboratory Manual, Chap. 14, pp. 553-612, 1988) or Samuel et al. (U.S. Pat. No. 5,242,799) (Examiner's point 5).

Applicants respectfully traverse the rejection of claims 49-66, 68-75 and 77, as Applicants do not believe that a *prima facie* case of obviousness can be made using the cited references.

The Examiner has cited Heilig et al. ("Heilig") as teaching a method using "an anti-thyroglobulin which is capable of binding to both types of thyroglobulin and further using an additional which is capable of binding a sugar chain structure on only one of the two types of thyroglobulins."

Applicants respectfully disagree. This reference describes monoclonal antibodies prepared against human thyroglobulin (hTg). Six mAbs were obtained. A two-side immunometric assay for hTg involved fixing on mAb to a microtiter plate and using a second, labeled, mAb for detection. The reference compares this assay to a conventional radioassay for Tg, finding that in 3 of 13 patients, the correlation was poor.

Applicants therefore disagree with the Examiner's contention and believe that the reference does not in any way suggest discrimination between two types of thyroglobulin in a sample, or that two types of thyroglobulin might be present in single sample. The reference only suggests that "the molecule" of hTg (see line 17 of the reference) might have different epitopes, which is quite common for a single protein species.

The reference states that "it might be worthwhile to use monoclonal antibodies to look for tumor-specific Tg species." Applicants note that this provides merely an invitation to experiment further, but does not indicate that any such species are even known. This reference is therefore does not provide any enabling teaching with regard to the recited methods for determining malignancy, claims 68-75 and 77.

Additionally, Applicants note that Heilig et al. does not discuss lectins, and therefore provides no disclosure or suggestion for the lectins recited in the claims.

Therefore, the citation of Heilig et al. does not add provide disclosure or suggestion of any additional steps of the present claims over the references cited in Examiner's point 4. We do not believe that a *prima facie* case of obviousness is possible using these references, and Applicants believe that claims 49-66, 68-75 and 77 are novel and non-obvious over Heilig et al., Hanham et al., Voller et al., Harlow and Lane and Samuel et al., taken separately or in combination.

Claims 49-66, 68-75 and 77 are rejected under 35 U.S.C. 103(a) as unpatentable over Wang et al. (Chung-hua Ping Li Hsueh Tsa Chin, vol. 19(2), pp. 90-93) in view of Lo Gerfo et al., (Lancet (1977), vol. 1, No. 8017, pp. 881-882), and further in view of Voller et al. (Rul. World Health Organ., Vol. 53, pp. 55-65, 1976) or Harlow and Lane (Antibodies, a Laboratory Manual, Chap. 14, pp. 553-612, 1988) or Samuel et al. (U.S. Pat. No. 5,242,799) (Examiner's point 6).

The Examiner cites Wang et al. ("Wang") as teaching a method using an anti-thyroglobulin antibody which is capable of binding to two types of thyroglobulin and further using a lectin which

is capable of binding to a specific sugar on only one of the two types of thyroglobulin. The Examiner appears to argue that although Wang teaches detection in tissues, Wang's method would be applicable in serum and is applicable to diagnosis of cancer.

Applicants respectfully traverse this rejection of claims 49-66, 68-75 and 77, as Applicants believe that a *prima facie* case of obviousness cannot be made using these references.

Applicants respectfully disagree with the Examiner regarding the teaching of Wang. Wang (Abstract) discusses lectin distribution in thyroid carcinoma cases, and indicates a "distribution of lectins" among different thryoid carcinoma types. However, based on the Wang abstract, Applicants do **not** believe that Wang teaches use of an anti-thyroglobulin antibody and a lectin to distinguish thyroglobulins. Applicants note the following points about Wang:

1) Wang discusses a difference in "lectin distribution" between different thyroid carcinoma types. This presumably refers to use of lectins in staining the carcinomas, but the abstract only implies that **where** the lectins stain in the tissue sample differs between different cancers. There is no indication in the abstract as to what molecules the lectins are binding to.

2) Wang discusses a correlation between the lectin distribution and Tg immunoreactivity. However, this does not indicate that the lectins are be binding to the Tg.

3) Thus, there is only a suggestion that Wang has simultaneously exposed fixed tissue samples to both lectin and an anti-thyroglobulin antibody. This would not allow distinction between different types of thyroglobulin present, since lectins may bind to other proteins as well. This therefore does not provide a suggestion for the addition steps recited in the claims.

4) Wang's assay would not allow measurement of the amount of thyroglobulin present as recited in the claims.

The Examiner has cited the LoGerfo reference as showing that the detection of thyroglobulin in serum and subsequent correlation to cancer is well known in the art. Applicants concur, and the present application noted this point on page 1, second paragraph of the specification. However, there appears to be no teaching or suggestion in LoGerfo regarding antibodies directed at two types of thyroglobulin as recited in the claims. Lectins do not appear to be mentioned in the reference.

Therefore, the citation of Wang et al. does not add provide any additional disclosure or suggestion of steps of the present claims over the references cited in Examiner's point 4 and 5. Applicants do not believe that a *prima facie* case of obviousness is possible using these references, and Applicants believe that claims 49-66, 68-75 and 77 are novel and non-obvious over Wang et al., Lo Gerfo et al., Voller et al., Harlow and Lane and Samuel et al., taken separately or in combination.

Claims 49, 50, 52, and 57-65 are rejected under 35 U.S.C. 103(a) as unpatentable over Canfield et al. (WO 87/00289) in view of Voller et al. (Rul. World Health Organ., Vol. 53, pp. 55-65, 1976) or Harlow and Lane (Antibodies, a Laboratory Manual, Chap. 14, pp. 553-612, 1988) or Samuel et al. (U.S. Pat. No. 5,242,799) (Examiner's point 7).

The Examiner cites Canfield as using an "anti-thyroglobulin antibody which is capable of binding to both types of thyroglobulin and further using a lectin which is capable of binding a specific sugar chain structure on only one of the two types of thyroglobulins."

Applicants respectfully traverse this rejection, as Applicants believe that no *prima facie* case of obviousness can be made using the cited references.

Most significantly, Applicants note that Canfield WO'289 appears to be directed mainly to human chorionic gonadotropin (hCG), which is not the same as thyroglobulin. Applicants have attached two documents demonstrating this point (Gradwohl's Clinical laboratory methods and diagnosis, 7th ed., pp. 1576-1577 (1970) and Malthiery et al., Eur. J. Biochem. vol. 165, pp. 491-498 (1987).) The Examiner is apparently taking Canfield's appropriate lectin as the recited lectin of step (b) of claim 49, and Canfield's detectable antibody as the anti-thyroglobulin antibody of step (a). However, there appears to be no suggestion in the Canfield reference to modify the disclosed method to be applicable to thyroglobulin. There would also not appear to be any suggestion or motivation in the other references to modify the Canfield method to be applicable to thyroglobulin.

Secondly, Applicants believe that there is no analogue in Canfield of the recitation of claim 50, steps (b)(i), regarding measuring a total amount of conjugates. In claim 50, this step and step (b)(ii), measuring the amount of conjugates of the specific lectin or specific antibody, are both performed. Similar recitations occur in claims 58, 59 and 60. Applicants believe that Canfield's disclosed method does not suggest this measurement.

In addition, Applicants believe that there is no suggestion in Canfield for an analogue to the anti-thyroglobulin antibody-2 of claims 61, 62 and 65, which cannot bind thyroglobulin to which the specific lectin or the specific antibody is already bound.

Applicants therefore believe that claims 49, 50, 52, and 57-65 novel and non-obvious over Canfield et al., Voller et al., Harlow and Lane, and Samuel et al., taken separately or in combination.

Claims 49-66, 68-75 and 77 are rejected under 35 U.S.C. 103(a) as unpatentable over Canfield et al. (WO 87/00289) in view of Tarutani et al. (J. Biochemistry, vol. 98(3), 1985) or Wang et al. (Chung-hua Ping Li Hsueh Tsa Chin, vol. 19(2), pp. 90-93), or Heilig et al. (Endocrin. Suppl. 108(267), p. 151, 1985), and further in view of Voller et al. (Rul. World Health Organ., Vol. 53, pp. 55-65, 1976) or Harlow and Lane (Antibodies, a Laboratory Manual, Chap. 14, pp. 553-612, 1988) or Samuel et al. (U.S. Pat. No. 5,242,799) (Examiner's point 8).

Applicants respectfully traverse this rejection of claims 49-66, 68-75 and 77. In addition to the remarks above directed to Canfield and the other cited references, Applicants here address the additional teaching of Tarutani.

As understood by the Applicant, Tarutani is cited by the Examiner for its general teaching of variation in sugar chains of thyroglobulin and correlation to cancer. Applicants concur that Tarutani describes con A-gel column chromatography of human Tg, indicating that human Tg was heterogeneous with respect to affinity for con A. The reference also studied thyroid tumor Tg, and indicated that there were two separable types of Tg, one that had a strong affinity for lectins and one that had a weak affinity for lectins. The Tarutani paper also appears to indicate on p. 854 that the two separable types of Tg were both detectable by anti-human Tg serum.

However, several points are notable about Tarutani. First of all, on page 853, left column, lines 31-40, even at heavy loading only 74% of the adsorbed Tg is recovered from the con-A gel, and "as Tg adhered strongly to the column, it was difficult to elute completely from the column ..." That is, Tarutani's method does not clearly provide a quantitative assay for the adsorbed Tg. It

should be noted that Tarutani measures the concentration of Tg by means of ultraviolet absorption in the eluate from the column. This is not a specific method for determination of Tg and may be affected by non-thyroglobulin protein in the sample. Tarutani therefore does not teach or suggest the use of an anti-thyroglobulin antibody as in the present claims. Given this point and the lack of quantitation due to the strongly adhered Tg, Applicants do not believe that Tarutani's method could even be modified to provide an accurate ratio of the two types of Tg. That is, Tarutani does not enable a measurement of the Tg ratio of the present claims.

In addition, Applicants respectfully disagree with the Examiner that there is a suggestion in the reference that such a Tg ratio might correlate with the malignancy of a cancer. Accordingly, there is no suggestion to quantitatively measure this ratio and compare the result with a reference fluid sample, as recited in the claims directed to a method of determining malignancy.

Applicants have also above discussed the teachings of Wang, Hanham and Heilig, and do not believe that these references provide any suggestion to measure this Tg ratio. In particular, Heilig does not discuss two types of thyroglobulin at all. Likewise, Wang only discusses the spatial distribution of lectins in thyroid carcinoma samples and does not even clearly indicate that the lectins are binding to the Tg. Hanham discusses glycosylation of Tg modified with enzymes, but does not discuss two types of naturally occurring Tg.

Applicants therefore do not believe that there is a suggestion or motivation in the Tarutani, Wang and Hanham references for modifying the Canfield reference to be applicable to thyroglobulins.

Applicants also note that there appears to be no teaching in Tarutani or Canfield of a reagent comprising both a lectin and an antibody, and that claims 52-55 are therefore not suggested.

Moreover, as noted above, the Examiner has rejected claims including use of a second anti-thyroglobulin antibody which cannot bind to a thyroglobulin to which the lectin is bound, that is, claims 60, 61, 62, 65, 70, 71, 72, and 75. Applicants can find no suggestion for this step in Tarutani.

Applicants therefore believe that claims 49-66, 68-75 and 77 are novel and non-obvious over Canfield et al., Tarutani et al., Wang et al., Heilig et al., Voller et al., Harlow and Lane and Samuel et al., taken separately or in combination.

Claims 49-77 are rejected under 35 U.S.C. 103(a) as unpatentable over Canfield et al. (WO 87/00289) in view of Tarutani et al. (J. Biochemistry, vol. 98(3), 1985) or Wang et al. (Chung-hua Ping Li Hsueh Tsa Chin, vol. 19(2), pp. 90-93), or Hanham et al. (Biochemica et Biophysica Acta, Vol. 884, 1986) or Heilig et al. (Endocrin. Suppl. 108(267), p. 151, 1985), and further in view of Voller et al. (Rul. World Health Organ., Vol. 53, pp. 55-65, 1976) or Harlow and Lane (Antibodies, a Laboratory Manual, Chap. 14, pp. 553-612, 1988) or Samuel et al. (U.S. Pat. No. 5,242,799), and further in view of Larena et al. (Langenbacks Archiv fur Chirurgie, Vol. 381/2, pp. 102-113, 1996) (Examiner's point 9).

This rejection is respectfully traversed. In the rejection, the Examiner additionally cites the Larena et al. ("Larena") reference as teaching that Lewis-type sugar chains are known in the art to be useful for detection of malignancy. This reference is thus being applied additionally to claims 67 and 76 which were not rejected in Examiner's point 8.

Larena does generally discuss antigens labeled Lea, Leb, Le(x), etc., which apparently refer to Lewis type sugar chains as recited in the claims. However, this reference does not discuss these anitgens as part of thyroglobulin, and the Larena reference at best suggests assaying specific Lewis type sugar antigens in thyroid cancer. Given the lack of any teaching concerning thyroglobulin, Applicants believe that there is no clear way to combine Larena with the teachings of the other references, and that Larena does not provide a suggestion for the recitation of the present claims.

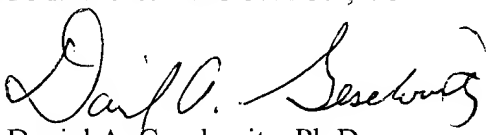
Given Applicants' above comments regarding the rejection in Examiner's point 9, Applicants believe that the additional teaching of Larena does not create a *prima facie* case of obviousness, and that claims 49-77 are novel and non-obvious over Canfield et al., Tarutani et al., Wang et al., Hanham et al., Heilig et al., Voller et al., Harlow and Lane, Samuel et al., and Larena et al., taken separately or in combination.

If, for any reason, it is felt that this application is not now in condition for allowance, the Examiner is requested to contact Applicant's undersigned agent at the telephone number indicated below to arrange for an interview to expedite the disposition of this case.

In the event that this paper is not timely filed, Applicants respectfully petition for an appropriate extension of time. The fees for such an extension or any other fees which may be due with respect to this paper, may be charged to Deposit Account No. 01-2340.

Respectfully submitted,

ARMSTRONG, WESTERMAN, HATTORI,
McLELAND & NAUGHTON, LLP



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Enclosures: Gradwohl's Clinical Laboratory Method and Diagnosis; Sam Frankel et al. (2 pages)
Primary Structure of Human Thyroglobulin deduced from the sequence of its 8448-base complementary DNA; Yves MALTHIERY et al. Eur. J. Biochem (5 pages)

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Volume 2

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Chapter 80

Tests for pregnancy

Alex C. Sonnenwirth

HUMAN CHORIONIC GONADOTROPIN

Human chorionic gonadotropin (HCG) is a hormone elaborated by the placenta, which is found in the blood, urine, amniotic fluid, colostrum, milk, and fetal tissues. The hormone appears soon after the first missed menstruation in human pregnancy, producing the "positive" reaction in pregnancy tests. It is also found when hormone-producing tumors are present, which can occur both in nonpregnant females and in males.[256] Two gonadotropic substances in the urine and blood of pregnant women were first demonstrated by Aschheim and Zondek in 1927[256]; they named these Prolan A (follicle-stimulating hormone, FSH) and Prolan B (luteinizing hormone, LH). Human chorionic gonadotropin was isolated finally in 1938 from placental cells grown in tissue culture. For the diagnosis of pregnancy, qualitative detection of HCG has been used since Aschheim and Zondek introduced their test for laboratory assay; quantitative determination of the hormone is used as an aid in the diagnosis of choriocarcinoma or hydatidiform mole and for investigation of pathologic pregnancies.

HCG is a glycoprotein with a molecular weight of approximately 80,000; it has a relatively high carbohydrate content in the form of galactose. It is quantitated in international units (IU) of gonadotropic activity. One IU is defined as the specific gonadotropic activity of 0.1 mg dried standard at the National Institute of Medical Research, London (First International Standard, 1938).[256] A Second International Standard has been established by the World Health Organization after stocks of the first standard ran low; the IU of chorionic gonadotropin was defined as the activity contained in 0.001279 mg of the Second International Standard (1964).[250]

In their concise review of pregnancy tests (1967),[400] Krieg and Henry pointed out that in evaluating pregnancy tests it is necessary to take into consideration (1) the specificity of the assay, i.e., does the test detect, and how often, other hormones such as pituitary gonadotropins or nonhormonal substances, resulting in false positive tests, and (2) its sensitivity, i.e., how many IU/ml are needed to result in a positive reaction. The physician should be informed as to what kind of test was performed, and he should be familiar with the specificity and sensitivity of the test.

For the diagnosis of hydatidiform mole or to evaluate disturbed or threatened abortion in the first trimester, quantitation of HCG should be performed. This can be done by determining the highest dilution at which the test remains positive. Results are often reported as the titer of the test (or various animal units in bioassays), but it is now customary to report results in IU/24 hr urine excretion or IU/L of first-voided morning urine. Random specimens should not be used for quantitative studies.

HCG levels[256, 399]

HCG becomes detectable in urine about the 24th day after last menstrual period (LMP). At the 40th day LMP, a level of about 5000 IU/24 hr is reached; it rises to a peak of 200,000 IU/24 hr or more any time between 50-90 days, but usually at about day 60-70.

The peak continues for about 10-20 days and then declines rapidly to about 4000-10,000 IU/24 hr. After delivery, HCG levels fall very rapidly. At 24 hr postpartum only small amounts are present, and at 72 hr HCG is no longer detectable in urine or serum.

In choriocarcinoma and hydatidiform mole, HCG titers are usually very high. The timing of the test is of importance because high levels of HCG must be differentiated from the normal peak described above. According to Hon,[256] a titer definitely higher than 500,000 IU/24 hr or a high titer that persists over 10 days (the usual duration of the normal peak) is indicative of these conditions. In incomplete or inevitable abortion, complete and missed abortion, and in ectopic pregnancies the HCG titers are usually very low (less than 3000 IU/24 hr urine).

Tests for detection and quantitation of HCG

Until 1960, detection and quantitation of HCG was performed by biologic assays, i.e., by demonstration of the biologic effects of HCG in a variety of animals. These tests are time-consuming and cumbersome; they involve maintenance of animals and are affected by individual and seasonal variation of animal sensitivity to HCG.

Beginning in 1960, various immunologic methods were described for the detection of quantitation of HCG. These are, at the least, comparable to bioassays both in sensitivity and specificity and are less costly, more easily standardized, and considerably more rapid than the bioassays. Some studies[239] actually indicate a greater accuracy of the immunologic methods as compared to the biologic methods. At the time of writing, the immunoassays have become widely accepted and have replaced the various biologic methods in many laboratories.

Both the biologic and immunologic methods are described below.

BIOLOGIC TESTS FOR PREGNANCY

An indispensable reference source for bioassays in pregnancy testing is Hon's *Manual of Pregnancy Testing* (1961).[256]

Aschheim-Zondek test[256]

This was the first test to be developed. It depends on the fact that HCG causes the formation of hemorrhagic follicles and corpora lutea in the ovaries of intact immature mice. Its disadvantage is that 5 days are required for its performance.

Inject subcutaneously five immature female mice (6-8 gm, 3 wk old) with urine in 6 portions divided over a period of 2 days (0.4 ml/injection for a total of 2.4 ml). Sacrifice the mice with ether or illuminating gas 4 days after the first injection and pin to a cork board. Open the abdomen and examine the ovaries. In most cases the diagnosis can be made macroscopically. In positive cases the ovaries are large, hyperemic, and show the so-called "blood spots" or hemorrhagic spots. If necessary, make microscopic sections. When results are positive, note graafian follicles, hemorrhage into the follicles, and corpora lutea.

Sensitivity: Approximately 1-6 IU HCG/ml.

"Quantitative" assay

This can be performed by injecting 1:10, 1:100, or higher dilutions of urine. The procedure is the same as in the qualitative test and results are reported as "A-Z positive in dilution 1:10," etc.

The qualitative test is quite reliable but it is not used generally because of the length of time and large number of animals involved. The quantitative test is only a gross estimate of the HCG present in urine.

Aschheim-Zondek test—Friedman modification[402]

In the Friedman test mature female rabbits are used. The rabbit should be isolated for 3 wk. Although ova continually mature and graafian follicles ripen in the ovaries of rabbits, ovulation does not take place until after copulation. Thus in properly caged female rabbits it is possible to study the effect of urine on ovaries previously free of corpora hemorrhagica.

1. Inject 10 ml urine into one of the marginal ear veins of the rabbit. Label the rabbit and keep in separate cage.
2. Sacrifice the rabbit 48 hr after injection. Examine the ovaries for ruptured hemorrhagic follicles.
3. Positive reactions are seen as follows: ovaries studded with 1-6 or more corpora hemorrhagica and a coiled hyperemic uterus. The small rosy spots that may appear in large clear follicles are suggestive but not positive, and another test should be made.

Sensitivity: 10-15 IU/ml urine.

Hogben test (South African clawed frog, *Xenopus laevis*)[402, 256]

This frog test is based on the peculiar biologic function of the mature female *Xenopus* in that she carries eggs throughout the year, extruding them only at mating or after the injection of hormones peculiar to pregnancy. Since the test animals are kept isolated from the males, the extrusion of any ova after the injection of suspected urine becomes definitive for pregnancy. The eggs can be seen with the naked eye.

Either serum or concentrated urine may be used.

Serum:

Obtain 20 ml blood, let it clot, centrifuge, and remove serum. Inject 3 ml serum into each of 2 frogs.

Select the dorsum of the animal as the site of injection, since it is the largest lymph space. Care as well as experience is required when injecting the frog. Puncturing the lung usually means death of the animal.

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Primary structure of human thyroglobulin deduced from the sequence of its 8448-base complementary DNA

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The mRNA encoding human thyroglobulin has been cloned and sequenced. It is made up of a 8301-nucleotide segment encoding a preprotein monomer of 2767 amino acids, flanked by non-coding 5′ and 3′ regions of 41 and 106 nucleotides, respectively. This preprotein consists of a leader sequence of 19 amino acids, followed by the sequence of the mature monomer, corresponding to a polypeptide of 2748 amono acids (M_r = 302773). On its amino-terminal side, 70% of the monomer is characterized by the presence of three types of repetitive units. In contrast, the remaining 30% of the protein is devoid of repetitive units. This last region however shows an interesting homology (up to 64%) with the acetylcholinesterase of *Torpedo californica*. The sites of thyroid hormones synthesis are clustered at both ends of the thyroglobulin monomer. By contrast, the potential glycosylation sites are scattered along the polypeptide chain.

Thyroglobulin is a protein specifically synthesised by the thyroid gland, and constitutes the support for the production of the two thyroid hormones, thyroxine and triiodothyronine [1]. The existence of thyroglobulin was demonstrated a century ago [2], but its structure has been elucidated only recently. It is a dimeric glycoprotein with an M_r of 660000, of two identical subunits [3, 4] encoded by a single mRNA with a sedimentation coefficient of 33 S (8500 nucleotides) [5–7]. Thyroglobulin is synthesised by the thyrocyte, then exported to the vesicular lumen where its maturation begins by the iodination of several tyrosine residues, and coupling of some of the iodotyrosine residues [8]. Then, by an endocytotic process, the molecule is absorbed into the thyrocyte where several selective cleavages occur in the lysosomes, resulting in the release of the thyroid hormones, and complete degradation of the rest of the molecule.

For a thyroglobulin iodine content of 0.5% (which is rarely attained in man) a maximum of 3.5 hormonal residues per thyroglobulin molecule are formed through a reaction catalyzed by the enzyme thyroid peroxidase [9]. Four hormone-synthesis sites have been described, corresponding to four tyrosine residues in fixed positions [10–12].

The structure of human thyroglobulin seems to be responsible for the specific fixation of iodine, and the production of functional thyroid hormones. Several human pathologies are associated with an abnormal thyroid function. Since the recent demonstration of the implication of a defect in thyroglobulin gene structure in the development of congenital goitre in cattle [13], it is likely that knowledge of the structure of human thyroglobulin mRNA will help to elucidate the structural bases of human thyroid pathologies. We describe here the complete nucleotide sequence of human thyroglobulin mRNA.

MATERIALS AND METHODS

Preparation and sequencing of DNA

cDNA fragments corresponding to human thyroglobulin mRNA were prepared from recombinant plasmids named M1–M4 and B2–B4 (see Fig. 1), as previously described [7, 14]. Two additional clones, named B1 (kind gift of H. Brocas and G. Vassart) and M5, were constructed by G-C tailing of cDNA, and eventual insertion into the *Pst*I site of pBR322, according to Maniatis et al. [15]. Restriction endonucleases were used as recommended by the suppliers. Fragments carrying 5′-protruding ends were labeled using alkaline phosphatase (CIP Boehringer) and T4 polynucleotide kinase (BRL) with [^{32}P]ATP (3000 Ci/mmol, Amersham). Fragments carrying 3′-protruding ends were labeled with cordycepin (3′-deoxyadenosine) or 2′,3′-dideoxyadenosine 5′-[^{32}P]-phosphate ([^{32}P]ddATP, 3000 Ci/mmol, Amersham) in the presence of terminal deoxynucleotidyl transferase (BRL).

The labeled fragments were isolated and sequenced according to the method described by Maxam and Gilbert [16].

mRNA preparation

Human thyroglobulin mRNA was extracted by the guanidine · HCl procedure [17] or by the guanidinium thiocyanate/CsCl gradient procedure [18], from a Graves' disease thyroid obtained surgically. A single passage through oligo(dT)-cellulose was used to prepare the fraction enriched in poly(A)-containing RNA. The quality of the RNA preparation was monitored by electrophoresis on agarose/methylmercury-hydroxide gels.

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† This paper is dedicated to the memory of Professor Serge Lissitzky.

Enzymes. Reverse transcriptase or RNA-directed DNA nucleotidyltransferase (EC 2.7.7.49); terminal deoxynucleotidyltransferase (EC 2.7.7.31); T4 polynucleotide kinase (EC 2.7.1.78); alkaline phosphatase (EC 3.1.3.1); restriction endonucleases *Bgl*II, *Eco*RI, *Pst*I and *Sau*3A I (EC 3.1.21.4).

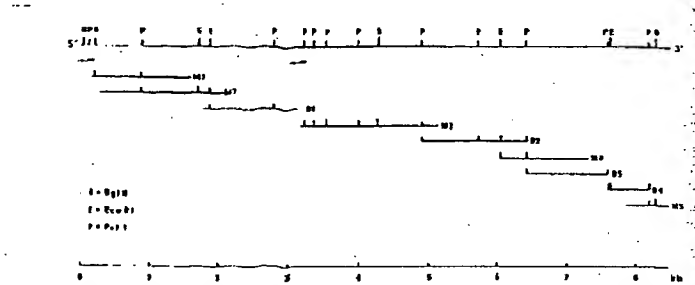


Fig. 1. *Restriction map of DNA coding for human thyroglobulin obtained from nine scored cDNA clones.* The sequencing of the 5′ end and the region between clones B1 and M3 were obtained by primer-extension. The primers were isolated from clones M1 and M3, respectively.

Primer-extension experiments

The 5′-end extension of clone M1 has been already described [19]. A gap between clones M3 and B1 was filled up by primer-extension: a 147-bp restriction fragment corresponding to position 35–182 of clone M3 was isolated by polyacrylamide gel electrophoresis, and subsequently cut by *Sau*3A1 (position 131 of M3). The fragment was labelled with ^{32}P at the 3′ end as described above, and the two complementary strands separated on a 8% acrylamide/urea gel, and eluted according to Maxam and Gilbert [16].

Poly(A)-rich mRNA (50 µg) was hybridized with 50 ng single-stranded primer DNA labeled at its 5′-end (1:1 ratio of homologous sequences) in 100 µl 0.4 M NaCl, 10 mM Pipes pH 6.4 for 4 h at 65°C. Ethanol precipitation was performed following hybridization. Extension was carried out for 45 min at 42°C in 0.1 M Tris/HCl pH 8.3, 0.14 M KCl, 10 mM $MgCl_2$, 28 mM 2-mercaptoethanol in the presence of 1 mM dATP, dCTP, dGTP, dTTP and 40 units of reverse transcriptase (PL) in a final volume of 50 µl. The mRNA was then hydrolysed by addition of 25 µl 0.15 M NaOH for 60 min at 65°C. The reaction was stopped by neutralization and the ss-cDNA was precipitated by cold ethanol. Sequencing of the ss-cDNA was performed according to Maxam and Gilbert [16].

Sequence analysis

Results were analyzed using several computer programs developed by B. Jacq and B. Delon (unpublished) and R. Lathy et al. [20]. These programs allowed the restriction analysis of nucleic acid sequences, the search for homologies in nucleotide and protein sequences and the study of hydrophilicity.

RESULTS

The complete nucleotide sequence of the human thyroglobulin mRNA was deduced from the sequence of nine overlapping recombinant plasmids, corresponding to 98% of the mRNA sequence, and for the remaining 2% by sequencing: primer extensions, corresponding to a gap between two clones, and to the 5′-end of the message (Fig. 1). The nine clones were inserted into the plasmid vector pBR322, by G-C tailing (clones M1–4 [7] and B1) or by ligation of cohesive ends (clones B2-4 [14]). Restriction mapping of the inserts and cross-hybridization experiments allowed the respective positioning of the inserts. The sequences were established unambiguously by sequencing both DNA strands. The regions could not be cloned: a 56-nucleotide fragment between clones B1 and M3, and a 156-nucleotide fragment at the 5′ end of the mRNA. Those regions were covered by primer extension, as described in Methods. The sequence of the latter was verified by sequencing an appropriate genomic clone and by comparison to the sequence published by others [21]. The sequence around the junction between clones B3 and B4 was monitored by sequencing a genomic subclone covering this region (generous gift of F. Baas).

The mRNA encoding human thyroglobulin is a single-stranded nucleotide chain of 8448 nucleotides, provided with a polyA tail. It consists of a 5′ non-translated segment of 108 nucleotides, followed by a coding sequence of 8301 nucleotides with a single open reading frame, and a 3′ non translated segment of 108 nucleotides, showing the canonical polyadenylation signal AAUAAA in position 8427 (Fig. 2). A polypeptide sequence of 2767 amino acids can be deduced from the nucleotide sequence. A leader peptide of 19 amino acids is followed by a 2748-amino-acid polypeptide corresponding to the monomeric human thyroglobulin. The deduced M_r of that polypeptide (302773) agrees with the size of the polypeptide obtained by *in vitro* translation of human thyroglobulin mRNA [22] and with the size of the glycosylated monomeric thyroglobulin [23].

The amino-acid composition is similar to that of the mature protein [24], and confirms a rather high amount of serine and glycine residues (9.7% and 7.6% respectively), and a small proportion of lysine (3.3%; Table 1). Hydrophobic and charged amino-acid residues are homogeneously distributed on the polypeptide, whereas the cysteines involved in the repetitive structures (see below) and for the tyrosines are not. Each monomeric chain contains 67 tyrosine residues representing 2.44% of total. They are mostly clustered in the repetitive structures and in a region near the C-terminal, where they constitute up to 9% of total amino acids.

20 potential *N*-glycosylation sites (Asn-Xaa-Thr/Ser) are present in the protomeric sequence, that is twice the amount

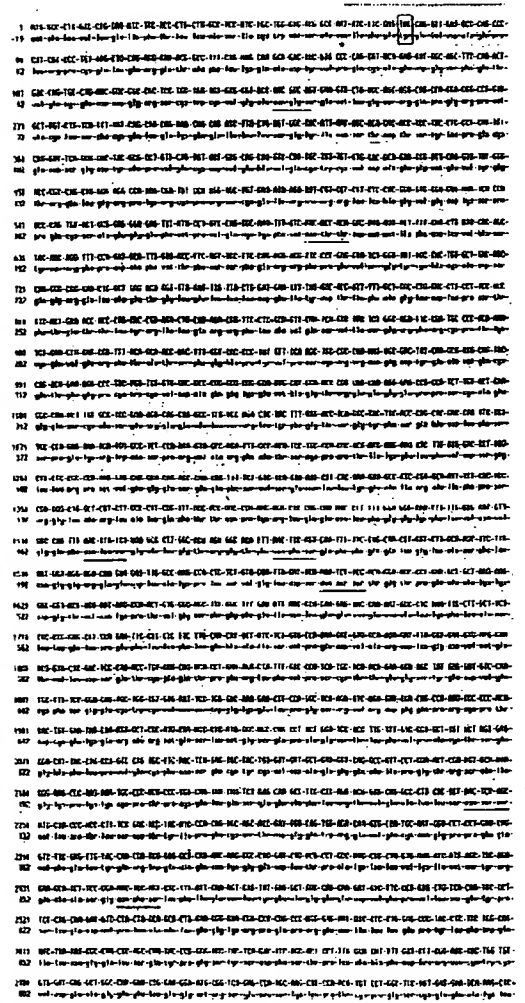


Fig. 2. *Nucleotide sequence of human thyroglobulin cDNA, and the deduced sequence of the protein.* Numbering starts at nucleotide 42, corresponding to the initiation codon ATG, and at the 20th amino acid residue (Asn), which is the N-terminal residue of the mature protein (not). The first 19 residues constitute the leader sequence of thyroglobulin. The AATAAA polyadenylation signal is underscored. The five *N*-glycosylation sites (Asn-Xaa-Ser/Thr) are underscored. The tyrosine residues involved in hormone synthesis are boxed

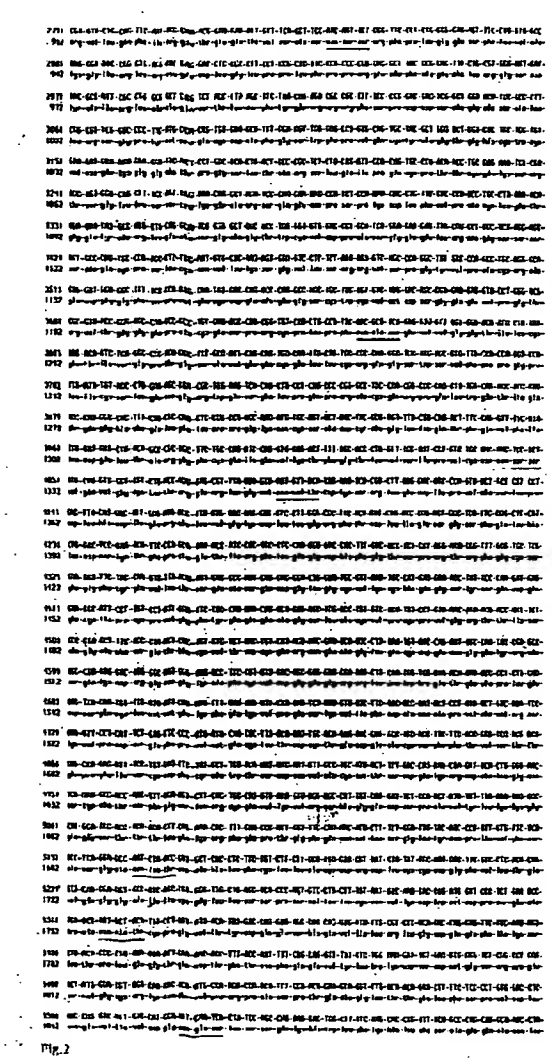


Fig. 2

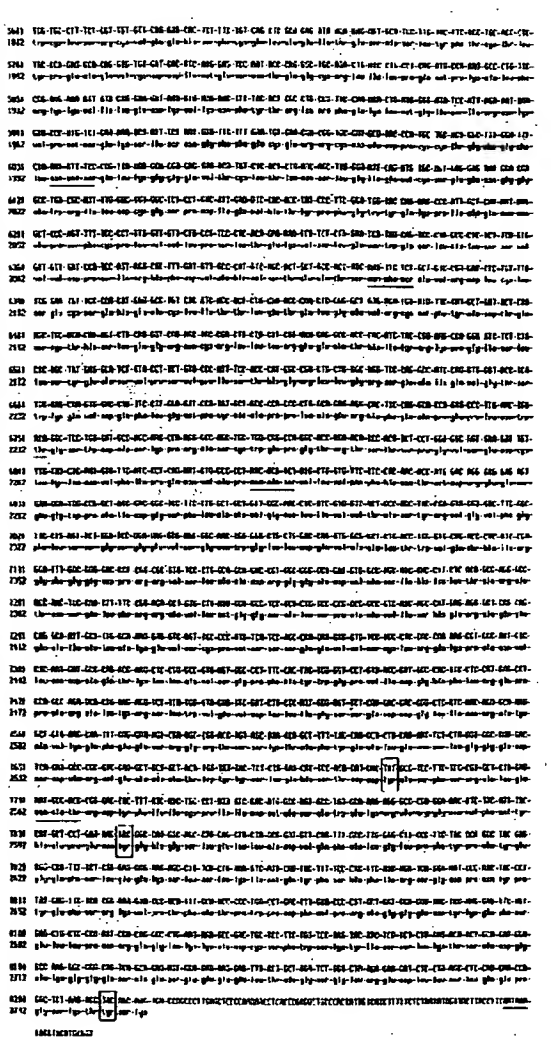


Fig. 2

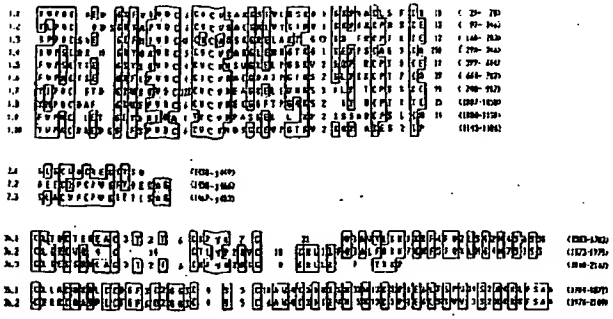


Fig. 3. *Internal homologies in human thyroglobulin primary structure.* The three types of homologous domains are aligned. Insertions/deletions have been introduced to optimize homology. Conserved amino acids are boxed according to the following scheme: for type 1 repeats, the amino acids should be present at least five times; for type 2 and type 3 repeats, at least twice. Residues showing no conservation between the domains are represented by numbers. Localization of the domains is shown on the right (the numbering is as in Fig. 2)

Table 1. *The amino acid composition of the human thyroglobulin monomer*

	Amino acid	Number of residues	Percentage
A	Alanine	195	7.38
R	Arginine	251	9.50
D	Aspartic acid	129	4.51
B	Asparagine	83	3.02
C	Cysteine	122	4.49
E	Glutamic acid	167	6.11
Q	Glutamine	171	6.25
G	Glycine	297	7.68
H	Histidine	41	1.49
I	Isoleucine	81	2.95
L	Leucine	258	9.39
K	Lysine	71	2.51
M	Methionine	27	0.95
F	Phenylalanine	108	3.97
P	Proline	171	6.22
S	Serine	257	9.32
T	Threonine	146	5.31
W	Tryptophan	43	1.56
Y	Tyrosine	67	2.44
V	Valine	142	5.20

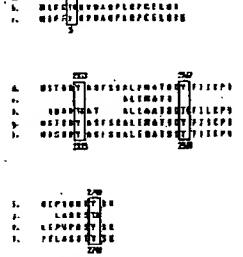
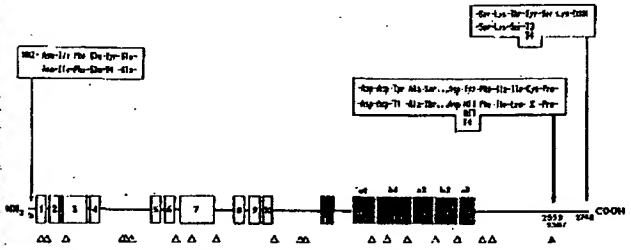


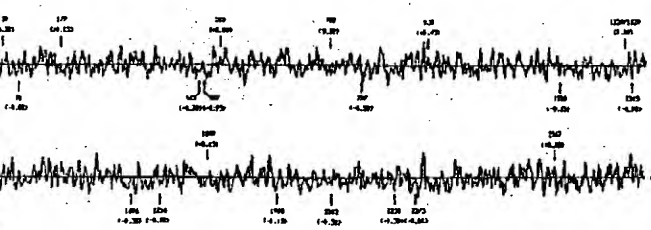
Fig. 4. *Hormonogenic domains in human thyroglobulin.* The hormonogenic domains identified to date in various species are represented by the one-letter code and are aligned for optimal homology. The four hormonogenic tyrosines are boxed and their positions in the protein are indicated. a, d, f, peptide sequences derived from the present work; b, four identical hormone containing peptides isolated from human, bovine, ovine and porcine thyroglobulins [2, 12, 95]; c, h, l, protein sequences derived from the bovine cDNA sequence [28]; the C-terminal region of the ovine thyroglobulin identical to that in sequence 1 (Durand, F. and Malthiery, Y., unpublished); g, k, protein sequence deduced from a partial rat cDNA sequence [36]; *f*, sequence of three peptides obtained from porcine thyroglobulin [10]; i, sequence of a hormone-containing peptide isolated from porcine thyroglobulin [11]; e, tryptic peptide, isolated from human thyroglobulin, containing a glycosidic chain attached to the asparagine residue [25]

of sites actually glycosylated in the mature protein. One of them was naturally displayed in human thyroglobulin, at position 2562 [25].

Analysis of the deduced amino-acid sequence of the protomer reveals the existence of three types of repetitive domains. Type 1 is exclusively present in the N-terminal portion of the molecule, and is repeated ten times between positions 29 and 1196. It is composed of approximately 90 amino acids, in which the positions of Cys, Pro and Gly residues are highly conserved. The proportion of Cys and Tyr in the type 1 domains is high, as compared to the entire protomer. Some insertions of variable length are found in fixed positions in the area, but seem to have no relation with the repeated domains. The second repeated domain is shorter (14–17 residues) and is present in triplicate between positions 1436 and 1438. The third domain (type 3) is repeated five times, between positions 1603 and 2186. As in type 1, maximal homology is observed near the Cys residues, whose positions are highly conserved. A more refined analysis of the type 3 domains allows one to distinguish two subtypes (3a and 3b; Fig. 3), suggesting that type 3 region originates from tandem duplication of an ancestral gene segment.



Schematic representation of the human thyroglobulin monomer. The ten domains of the type 1 homology are represented by open boxes. The three domains of the type 2 homology are represented in black, in the middle of the molecule. The type 3 homology (hatched boxes) is composed of subtypes 3a (a1, a2, a3) and 3b (b1, b2). The triangles correspond to potential glycosylation sites; the closed triangle corresponds to the actual glycosylation site (see Fig. 4). The four hormonogenic sites are indicated by arrows; in the boxes, the upper line shows the sequence deduced from the nucleotide sequence, the bottom line corresponds to the peptide sequence characterized on the mature human or porcine thyroglobulins (see Fig. 4). MIT = monoiodotyrosine; DIT = diiodotyrosine; T3 = 3,5,3'-triiodothyronine; T4 = thyroxine



Hydrophilicity profile of the human thyroglobulin monomer, starting from the N-terminal of the molecule. The computer program used for hydrophilicity coefficients of the amino acids are from Hopp and Woods [26], using a five-amino-acid window. Figures indicate the position of the potential *N*-glycosylation sites (Asn residues in the tripeptide Asn-Xaa-Ser/Thr) and the corresponding hydrophilicity indexes, as a measure of the hydrophilicity of the environment of that amino acid [26]

DISCUSSION

The analysis of the amino-acid sequence of human thyroglobulin, as deduced from the complete sequence of its cDNA, reveals that the 2767 amino acid protomer consists of three regions with distinct structural characteristics. Within the 200 amino-terminal residues of the protein, three families of homologous domains could be detected, for a total of 18 repeats (Fig. 3). By contrast, no internal homologies could be detected within the 580 C-terminal residues. A low cysteine content and a high tyrosine proportion (up to 9% of total residues between the positions 2539 and 2671) further distinguishes the C-terminal from the rest of the protomer. Those structural differences might be related to functional differences, since Marriq et al. [10] and Rawitch et al. [12] demonstrated that four of the tyrosine residues involved as 'donor' sites in thyroxine and triiodothyronine formation are situated in positions 5, 2553, 2567 and 2746 of the mature protein (Fig. 4). If the central portion of the molecule does not contain hormonogenic sites, it could play a structural role, even in the positioning of the hormonogenic tyrosines, or in the endocytosis of thyroglobulin. Unfortunately, analysis of variations in hydrophobicity along the protomer, performed according to Hopp and Woods [26], showed no important differences between the three domains. It is noteworthy however that the only *N*-glycosylation site whose position has been determined (2562) is part of a hydrophilic domain (Fig. 6). Since approximately half of the 20 potential glycosylation sites are actually glycosylated in the mature protein, and since 8 sites out of 20 potential residues show a positive hydrophilicity index, the search for glycosylated residues should begin with these sites [27].

An organisation similar to that of human thyroglobulin was recently reported for bovine thyroglobulin [28], including the same number of internal repeats in the central portion of the molecule. The bovine mRNA is slightly shorter, but the corresponding protomer is two amino acids longer. Actually, the bovine protomer shows six single amino-acid alterations, as compared to the human protomer, two deletions in positions 440 and 1799, and four insertions in positions 792, 985, 1422 and 1450. Homology between the two mRNAs is 81.25% (81.75% on the coding region) and 77.22% between

the protomers, but increases in the hormonogenic regions, up to 100% for the 20 N-terminal residues (see Fig. 4). The strong homology between human and bovine thyroglobulin mRNAs, together with data on the structure of the human thyroglobulin gene suggest that the three domains observed in the mRNA structure might be evolutionally different. In the central region, homologies of types 1 and 3 probably originate from multiple duplications.

Computer searching in a data bank for homologies between the type 1 repeat and fragments of other proteins revealed that a tripeptide Cys-Trp/Tyr-Cys, whose position is highly conserved in all 10 cases of the repeat, was found in all known scorpion neurotoxins. Neurotoxins are short polypeptides of about 60 amino-acid residues [29], in which the tripeptide helps to maintain a strict tridimensional structure, but its role in thyroglobulin is unknown. Furthermore, the distribution of the other cysteine residues in the type 1 repeat and in neurotoxins is similar. More intriguing is the homology found between the C-terminal (non-repetitive) end of thyroglobulin and *Torpedo californica* acetylcholinesterase, as described by Schumacher et al. [30]. The amazing 64% homology between segments 2314–2360 of human thyroglobulin and 147–197 of acetylcholinesterase is suggestive of a common function that was conserved during evolution. That function is unknown as yet, although several hypotheses have been put forward [31].

Knowledge of the structure of human thyroglobulin mRNA, and of the organisation of the corresponding gene [32], should facilitate the development of studies on the structural bases of defects in thyroglobulin production. Along this line, defect in the structure of bovine thyroglobulin mRNA has already been linked to the existence of a hereditary goitre [13].

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